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Research Article

Impact of biosynthesized copper oxide nanoparticles using Thuthi Keerai *Abutilon indicum* on Dotted molly, *Poecilia sphenops*

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Abstract
Recently, nanoparticles have been widely used in various sectors, including agriculture, which can lead to environmental toxicity. Copper is a crucial trace element for fish, playing essential roles in various metabolic processes. The present study aimed to investigate the impact of biosynthesized copper oxide nanoparticles using Thuthi Keerai (*Abutilon indicum*) on the haematological and biochemical characteristics of Dotted Molly (*Poecilia sphenops*). Copper oxide nanoparticles were synthesized using Thuthi Keerai and characterized by UV-visible spectroscopy, Scanning Electron Microscopy, energy-dispersive X-ray spectroscopy, X-ray diffraction, and Fourier Transform Infrared Spectroscopy. Various concentrations of CuO NPs, including 0 (control), 5, 10, 15, 20, and 25 ppm, were used at 96 hours for sublethal analysis on Dotted Molly. The concentration at which 50% fish mortality occurred was taken as the median lethal concentration. In UV-Vis spectroscopy, the CuO NPs were measured at a wavelength of 340 nm. SEM image was observed at the wavelength range of 5µm. The EDAX spectrum recorded three peaks located between 1 and 8 keV. In XRD, the different peaks were indexed as 59.94 (110), 31.9 (202), and 24.14 (311). The FTIR spectrum was analyzed in the range between 400 and 4000^{-1}. White blood corpuscles, haemoglobin, and red blood corpuscles were higher (7200 cells/cumm, 1 gm/dl, and 0.4 million/cumm) in fish exposed to 1 ppm of CuO NPs. Protein, carbohydrate, and lipid levels were decreased by 1 ppm. The results confirmed that CuO nanoparticles enhance blood parameters and affect the biochemical parameters of the Dotted Molly.

Keywords: Biochemical, Copper oxide, Dotted molly, Haematology, Nanoparticles, Toxicity

INTRODUCTION

Nanotechnology has garnered significant attention in recent years because the materials synthesized on the nanoscale exhibit different physical and chemical characteristics compared to bulk materials. This enables the incorporation of nanoparticles into various applications, including agricultural, medical, and environmental fields (Saratale *et al*., 2018; Mahmoud *et al*., 2020). Since nanotechnology is developing rapidly, the green production of metallic oxide nanoparticles using plant extracts has emerged as an environmentally benign technique that allows for control over the material's size, shape, and quality (Salem *and* Fouda, 2020). Green synthesis methods, which employ non-toxic solvents such as biological extracts, offer several benefits over chemical ones, including simplicity. This is why plant extracts-assisted CuO NPs have garnered considerable interest (Mekala *et al*., 2016). According to Farkas *et al*. (2002), fish are frequently employed to assess the condition of aquatic ecosystems due to the accumulation of contaminants in the food chain. Accordingly, measuring metal levels in fish is crucial for human health (Uysal *et al*., 2008). The global ornamental fish trade is estimated to be worth US$18-20 billion, supported by approximately 100 million hobbyists worldwide and has been expanding at a rapid pace in recent years. India has considerable potential in the production and trade of ornamental fish due to the rich biodiversity of species hailing from diverse aquatic ecosystems, a favourable climate and the availability of a huge pool of low-cost labour. There are approximately 5,000 ornamental fish producing units spread across the country, with about 80% based on freshwater, while the rest are from brackishwater and marine (Shinoj *et al*., 2021). One of the most beautiful ornamental fish is *Poecilia sphenops*. Rich phytochemicals and antioxidants found in the aqueous extract of *A. indicum* leaves serve as capping and reduction agents for the environmentally friendly manufacture of copper oxide nanoparticles (Amal Sabour *et al*., 2022). The present study was carried out to synthesize CuO NPs using *A. indicum* leaf extract and to evaluate the obtained CuO NPs for their toxicity against the dotted molly fish, *Poecilia sphenops*.

MATERIALS AND METHODS

Collection of Thuthi Keerai *Abutilon indicum* Leaf

The Thuthi Keerai, *Abutilon indicum,* is a medicinal plant of the family Malvaceae that has heart-shaped leaves with sharp ends and small yellow flowers. The plant grows up to 2 meters in height and is a perennial shrub. Thuthi leaves are believed to have enormous medicinal value. Thuthi Keerai is used in traditional medicine for its demulcent, aphrodisiac, and laxative properties. The plant is also used for its antibacterial and anti-inflammatory properties, and can be used to treat wounds. The leaves of Thuthi Keerai (*A. indicum)* were collected from the local places around Gandhigram, Tamil Nadu, India.

Preparation of *Abutilon indicum* aqueous leaf extract

To prevent microbiological contamination, 10 g of fresh *A. indicum* leaves were surface sterilized with 0.1% $HgCl_2$ for one minute after being twice cleaned with distilled water (Kalra, 1998). After being finely chopped, the leaves were heated for 15 minutes at 60 °C in 100 millilitres of distilled water. After passing through the Whatman number 1 filter paper, the mixture was refrigerated at 4°C for later use.

Synthesis of copper oxide nanoparticles using the aqueous leaf extracts of *Abutilon indicum* (Ai-CuO NPs)

In a 500 mL beaker, the copper sulphate pentahydrate solution and the aqueous extract of *Abutilon indicum (*4:1 v/v) were heated to 80^{0}C °C while being continuously stirred for 25 minutes. To settle, the mixture was left in the dark for 24 hours. To purify the mixture, centrifugation was performed three times at 6000 rpm for five minutes. Any remaining biological extract was then dispersed using deionized water, and the buff precipitate was repeatedly washed with ethanol. Lastly, the precipitate was dried at 100°C for two hours in an oven (Sivakumar *et al*., 2024).

Characterization of CuO Nanoparticles

UV-Vis Spectroscopy

The absorbance spectra of the reaction medium were measured after 30 minutes to observe the reduction of Cu ions. The sample's most significant absorbance spectra of *Ai*-CuO NPs were obtained at 300–500 nm after approximately 1 mL was taken for UV–Vis spectrum analysis (Rahul *et al*., 2025).

Scanning Electron Microscopy (SEM) and Energy Dispersive X-ray (EDX) analysis

The surface morphology and size of the nanoparticles were obtained by Scanning Electron Microscopy (SEM) analysis. The morphology and composition of the copper nanoparticles were examined by Scanning Electron Microscope (SEM) using a HITACHI SU600 equipped with energy-dispersive (Rahul *et al*., 2025).

X-ray Diffraction analysis (XRD)

Using an XRD 6000/6100 (Shimadzu Corporation, Nakagyo-ku, Kyoto, Japan), the particle size of Ai-CuO NPs was assessed. X-ray diffraction is one of the primary analytical methods for determining the phase of crystalline materials. The average bulk composition was ascertained after carefully grinding the sample under analysis. Debye-Scherrer's equation was used to determine the grain size of the AI-CuO NPs. The equation D ¼ 0:94l=b cos q is where l is the wavelength (Cu Ka), b is the CuO (101) line's full-width half maximum (FWHM), and q is the diffraction angle (Chan *et al*, 2024).

Fourier Transform Infrared Spectroscopy (FTIR)

Two milligrams of *Ai*-CuO NPs were combined with 200 mg of FTIR-grade potassium bromide and formed into a pellet for Fourier Transform Infrared (FTIR) spectroscopy. After placing the pellet into the sample holder, FTIR spectra were captured at a resolution of 4 cm-1 using FTIR spectroscopy (Thermo Scientific Nicolet-is5, Waltham, USA) (Rathi *and* Jeice, 2024).

Experimental Study

Collection of fish

Dotted molly (*P. sphenops*) (7.8±0.02g) were purchased from AG Fish farm, Kadachanendal, Madurai, Tamil Nadu, India and carried to the laboratory in polythene bags filled with oxygenated water. Fishes were acclimatized in lab conditions (60x45x45 cm) for 5 days at 28± 2^{0}C. During acclimation, the fish were fed with trainee feed containing wheat flour and tapioca flour.

Table 1. Estimation of lethal concentration value of fish exposed to copper oxide nanoparticles and its confidence limits

Probability	95% Confidence Limits for con			95% Confidence Limits for Log		
	Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
.010	2.176	.017	4.815	.338	-1.761	.683
.050	3.456	.096	6.414	.539	-1.018	.807
.100	4.423	.237	7.524	.646	-.626	.876
.150	5.224	.434	8.422	.718	-.363	.925
.200	5.962	.698	9.254	.775	-.156	.966
.250	6.678	1.045	10.084	.825	.019	1.004
.300	7.395	1.492	10.958	.869	.174	1.040
.350	8.127	2.061	11.923	.910	.314	1.076
.400	8.889	2.774	13.042	.949	.443	1.115
.450	9.693	3.648	14.411	.986	.562	1.159
.500	**10.556**	4.693	16.185	1.024	.671	1.209

Experimental set-up

For experimental studies, the glass tank (60 x 45 x 45) was filled with 15 L of water. A disparate concentration of copper oxide nanoparticles was then dispersed into the water with 5,10,15,20, and 25 ppm concentrations. Five healthy fish were introduced into the glass tank. A control tank, without CuO NPs, containing 15 L of water and five fish, was also maintained. Three replicates were kept for each concentration group. After 24 hours, the mortality and survival rates of the fish in the tanks treated with copper nanoparticles and the control were noted. It was determined that the median concentration (LC_{50}) for 24 hours was 25 ppm, at which 50% of the fish died. A comparable experimental design was also used to calculate the median lethal copper concentration for Dotted Molly over 96 hours. The median lethal concentration was calculated using the probit analysis (Finney, 1978).

Acute toxicity analysis (LC_{50})

Twenty-four hours before the start of the experiment, the fish were not fed to maintain their metabolic states at a reasonably stable level. Fish weight and length were noted from the beginning. The Organisation for Economic Cooperation and Development guidelines (OECD,1992) were followed by exposing the fish to biologically synthesized CuO NPs for 24, 48, 76, and 96 hours using the static method (the water was not changed during the test). Over 96 hours, the Dotted Molly was exposed to CuO NPs at varying concentrations, including 0 (control), 5, 10, 15, 20, and 25 ppm. Five fish were taken from each concentration in each experiment, which was conducted in a 15 L glass tank. Mortality was tracked throughout the experiment, and the LC_{50} of the CuO NPs was calculated using statistical Probit analysis using SPSS (Table 1).

Sub-acute toxicity analysis

Twenty-litre glass tanks were obtained, and fifteen litres of water were placed inside each tank. Five healthy fish weighing 7.8±0.02g each were placed in each glass tank with varying concentrations of CuO Nps (0.1, 0.2, and 1 ppm). Fish survival and manifestation were tracked over 14 days in each concentration. In every exposure, including the control, the behaviour of the fish was observed. The fish were sacrificed to study toxicological parameters at the end of the trial period.

Analysis of haematological parameters

Blood samples were taken from fish in each exposure. After collecting blood samples in a container containing the anticoagulant EDTA, the samples were placed on the haematology mixture machine for ten minutes, and then mixed. Then samples were given to the cell counter device to determine the blood cells, including white blood cells, red blood cells, platelets, haemoglobin, haematocrit, neutrophils and lymphocytes.

Analysis of biochemical parameters

The total protein was determined Spectrophotometrically at 660nm (Lowry *et al*., 1951). Total carbohydrate content was estimated using the Anthrone method (Carrol *et al*., 1959). Total lipid content was estimated by Folch *et al*. (1951).

RESULTS AND DISCUSSION

The visual appearance of a colour change from blue to greenish-brown precipitate first confirmed the synthesis of CuO NPs. Nehru and Tharani (2020) reported that the optical properties were studied by using UV-Vis Spectroscopy. Copper oxide nanoparticles' UV-visible absorption spectra were evaluated at 200 and 300 nm wavelengths. A high-intensity absorption peak was recorded at 280 nm for Ms-CuO NPs (Figure 1). The measurement of absorbance peak from 200 to 300 nm indicates CuO NPs. Throughout the process, distinct bands at 280 nm were observed, indicating the synthe-

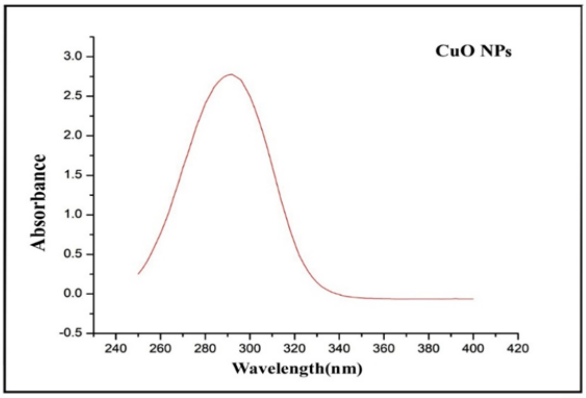

Fig. 1. *UV- Vis image of copper oxide nanoparticles*

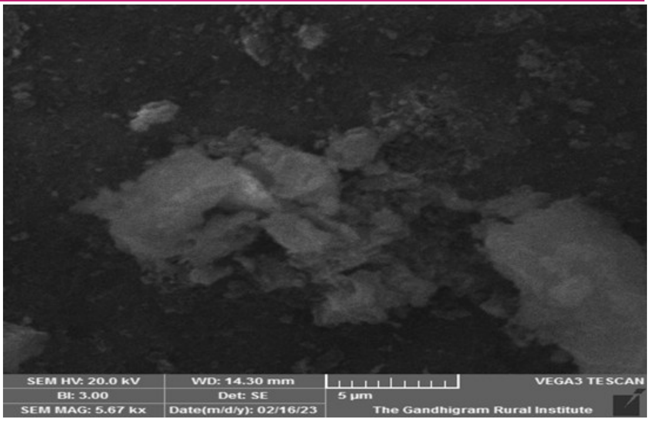

Fig. 2. *SEM image of copper oxide nanoparticles*

sis of CuO nanoparticles. To enhance the performance of the synthesized Cu O NPs in various applications, it is essential to understand their shape. Hence, scanning electron microscopy was used to examine the material's shape. According to SEM data, the produced copper oxide nanoparticles are spindle-shaped, contain agglomerated copper oxide elements, and range in size from 85nm (5µm) (Figure 2). According to Labaran *et al*. (2024) and Muthu Thiruvengadam *et al*. (2019), the SEM images of the synthesized Cu-NPs showed irregular particles grouped in clusters with a variety of structures, ranging from polygons to spherical shapes. The irregular shapes of the Cu-NPs made with *A. scholaris* show that the plant extract contains bioactive compounds that can effectively reduce and stabilize copper ions, resulting in the formation of nanoparticles with a variety of shapes.

Most of the nanoparticle sizes range from 25.19 nm to 28.73nm. According to the image, the sample contains nanoparticles of different sizes, which is consistent with the outcome of the chemical reduction procedure (Ayesha Khan *et al*., 2015). A similar result has also been reported in the SEM analysis of copper nanoparticles synthesized using aqueous leaf extract of *Vernonia amygdalina* plant(Ananda Murthy *et al*., 2021).

The composition of elements was identified by an energy-dispersive X-ray spectroscopy system (EDAX). The copper was 3.73 %, and the Oxygen was. 84% as recorded in EDAX analysis. (Figure 3). EDAX results revealed that the copper oxide nanoparticle peaks are located between the EDAX elements, which were analyzed on the surface of the obtained nanoparticles. EDAX spectrum recorded on the experimental sample is shown as three peaks located between 0.5 KeV, 8.0 KeV, and 8.04 KeV. The carbon and oxygen peaks in the samples verified the presence of carbon-based stabilizer (Ayesha *et al*., 2015). EDAX was used to characterize the elemental composition of the synthesized CuNPs utilizing *Pedalium murex* synthesized copper nanoparticles, as reported by Kannan *et al*. (2024). The XRD diffraction peaks were indexed as 59.94^0 (110), 31.9^0 (202), and 24.14^0 (311), as represented in Figure 4. All diffraction peaks were indexed according to the hexagonal phase of the copper nanoparticle. The XRD results were analyzed to assess the crystalline nature, and the average size of the peaks was 111, 002, and 202. An XRD analysis was performed on the crystal size. The XRD diffraction peaks were indexed as 111, 200, and 220, the characterized diffraction indices at 20

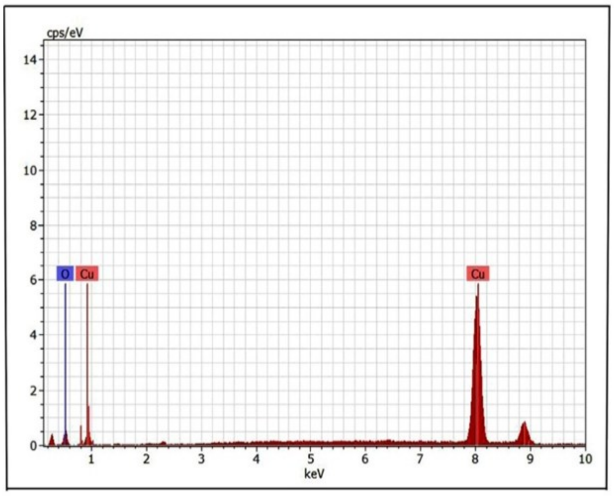

Fig. 3. *EDAX analysis of copper oxide nanoparticles*

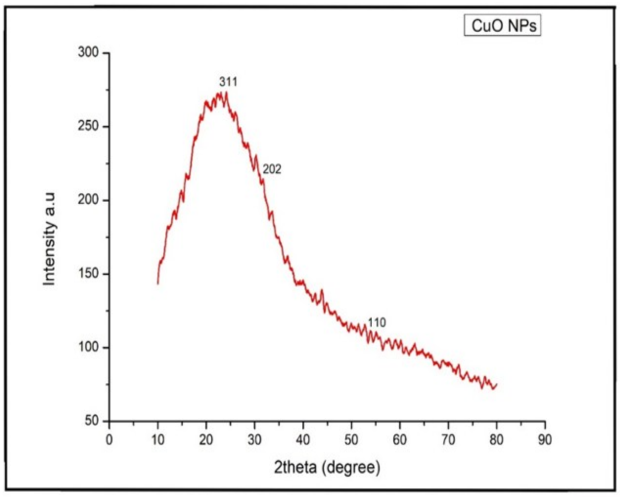

Fig. 4. *XRD Image of copper oxide nanoparticles*

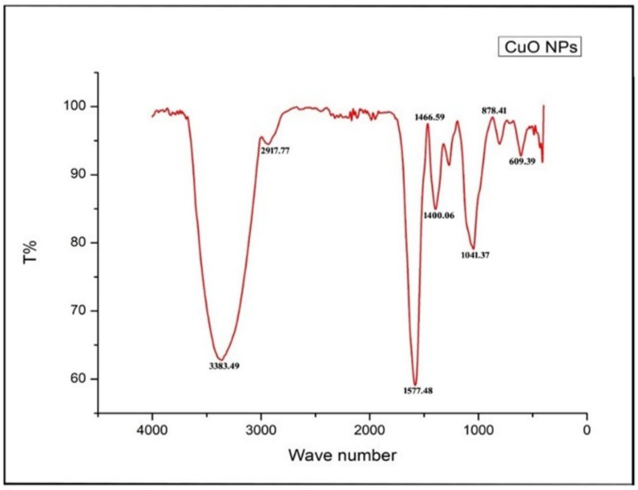

Fig. 5. *FTIR image of Copper Oxide Nanoparticles*

-value (Razium *et al*., 2013). A similar result was also reported by Suresh *et al*. (2016) in their XRD analysis of copper nanoparticles. Cu was discovered in the final products, with a peak at 1.3 keV verifying the presence of the necessary element. A similar observation was taken in this study, showing the XRD diffraction peak value. Fourier Transform Infrared Spectroscopy studies were performed to determine the potential functional groups responsible for reducing the copper ions in chemically produced copper oxide nanoparticles. Copper oxide nanoparticles' FTIR spectra were examined between 4500 and 500 cm^{-1}. The bands were observed in different peaks at 3383.49, 2917.77, 1577.48, 1466.59, 1400.06, 1041.37, 878.41, 609.39, were associated with 0-H alcohol, N-H amine salt, N-O nitro compound, C-H alkane, C-F fluoro compound, CO-O-CO anhydride-CI halo compound, C-I halo compound (Figure 5).

Haematological parameters have been used to quantify physiological changes in stressed fish, thereby providing a good indication for environmental monitoring, toxicological studies, and the detection of illness and stress (Seibel *et al*., 2021). Haematological parameters of Dotted molly exposed to CuO NPs for 14 days are presented in Table 2. All the haematological parameters increased with increased levels of CuO NPs. Likewise, Mekala and Rajan, (2023) found that when the quantity of chemically and biologically generated CuO NPs increased, the levels of hemoglobin, neutrophils, lymphocytes, eosinophils, RBC, WBC, and polymorphic were all considerably reduced in *Cyprinus carpio*.Yesudas *et al*. (2014) reported that haematological parameters of common carp exposed showed ionoregulatory interference, as well as compensatory responses, allowing the fish to maintain their haematology. The study showed that a change in exposure significantly affected the haematology of common carp. Ahmed *et al*. (2015) also reported that the copper sulphate in common carp altered haematological parameters, such as RBC, WBC, Hb, PCV, MCV, and MCH, while the Hb levels were slightly increased compared with the control fish. According to Noureen *et al*., (2022), exposure to CuO NPs resulted in a substantial decrease in the values for Hb, Hct, RBC, MCV, MCH, and MCHC, but a significant rise in the WBC and platelet count when compared to the negative control group. Following exposure to CuSO4, erythrocytes, hematocrit, hemoglobin, and lymphocytes all decreased significantly on all experimental days (Naz *et al*., 2023). Deshmukh *et al*. (2015) reported decreased haematological parameters, including RBC and WBC, blood glucose, and haemoglobin. According to Ramesh *et al*. (2014), the lower oxygen content in fish blood may be the cause of the lowered Hct value. Furthermore, toxicant stress on erythropoietic tissue is also indicated by reduced Hct values, which show cell shrinkage (Saravanan *et al*., 2011). Yahya (2023) found that while RBC and HT reduced with increased exposure to CuO nanoparticles, the mean values of Hb, MCV, MCH, and MCHC in the blood of Nile tilapia dropped in the serum of treated fish as the concentration increased.

The protein, carbohydrate, and lipid content in the gill, muscle, and liver of Dotted Molly, which was in control

Table 2. Haematological parameters of Dotted Molly exposed to copper oxide nanoparticles for 14 days

Parameters	Control	T1	T2	T3
WBC (Cells/Cumm)	2,500	4,200	6,700	7,200
Polymorph Neutrophils (%)	21	20	24	22
Lymphocytes (%)	66	60	62	68
Eosinophils (%)	13	10	14	10
Haemoglobin (gm/dl)	0.4	0.7	0.8	1.0
RBC Count (Million/cmm)	0.2	0.28	0.3	0.4
Haematocrit (PCV)	2	2.1	2.4	2.7
MCV (fi)	63	78	73	70
MCH (pg)	19	25	24	27
MCHC %	31	33	36	40
Platelets (Lakhs/cumm)	1.76	0.90	1.03	1.10

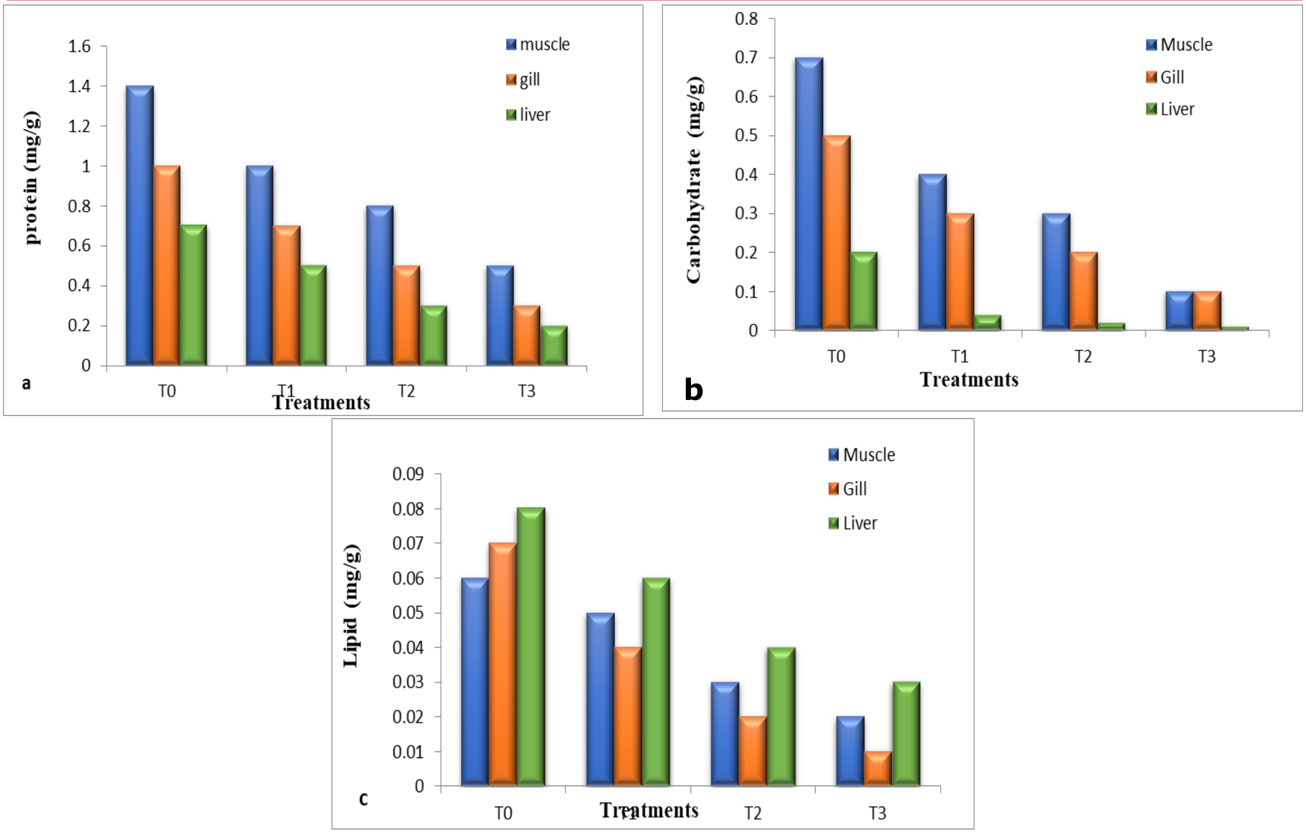

Fig. 6. *Total protein (a), carbohydrate (b) and lipid(c) of dotted molly exposed to 14 days of copper oxide nanoparticles*

and exposed to three different concentrations of CuO NPs, are presented in Fig. 6. The protein content in gill, liver and muscle decreased with increasing concentrations of copper nanoparticles. In comparison to the control group, a reduction in protein levels was noted in the muscle, gills, and liver of *Common carp* when the concentration of biologically produced CuO, Nps, rose (0.23-0.93 ppm) across exposure periods (Mekala and Rajan, 2023). Mehibeen Javad and Zazura Usmani (2014) reported an increased level of total protein, albumin and globulin in the liver and muscle of *Channa punctatus*. In the muscle of *Oreochromis niloticus,* increased protein was reported (El-Serafy *et al*., 2013). According to the current investigation, Dotted Molly treated with copper oxide nanoparticles showed reduced protein in its liver, muscle, and gills when compared to the corresponding control. Sadeghi *et al.* (2024) reported that total protein content was decreased with increasing concentration of copper oxide nanoparticles in *Carassius auratus*. The carbohydrate content in copper nanoparticles exposed fish decreased compared to the control group. According to other researchers, after prolonged exposure to *Labeo rohita*, serum glucose levels increased and then decreased until they were depleted (Zutshi *et al*., 2010). The lipid content in copper oxide nanoparticles exposed to *C. carpio* decreased when the concentration increased. A similar finding was observed when comparing the results to the reference: the total lipid levels were significantly decreased. Further studies also found that these factors have significantly increased (Vinodhini and Narayanan, 2008; Zutshi *et al*., 2010; Hanan *et al*., 2013). A change in lipid metabolism or a possible impairment in clearance from plasma, which favours liver disease, may cause an elevation or depletion in the lipid profile. A concentration-based increase and decrease of protein, lipid and carbohydrate in air-breathing catfish *Mystus cavasius* (Kesavasasus *et al*., 2011).

Conclusion

This study assessed the impact of biosynthesized CuO nanoparticles using Thuthi Keerai *Abutilon indicum* on the haematological and biochemical parameters of the ornamental fish Dotted Molly. The results concluded that the haematological parameters increased with increased concentration, while the biochemical parameters decreased with increased concentration of CuO nanoparticles in the rearing water of Dotted Molly. The key novelty of this investigation is the use of biosynthesized CuO NPs on the impact of haematological and biochemical parameters of the commercially important ornamental fish Dotted Molly.

Conflict of interest

The authors declare that they have no conflict of interest.

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